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A STUDY OF TRYPANOSOMA AMERICANUM

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On using some cattle blood for other work, the writer encountered the very large haemoflagellate first described by Crawley in 1909 under the name of *Trypanosoma americanum*. Later in 1912, Crawley more fully described the morphological, cytological and cultural characters of this interesting form. Since the appearance of Crawley's publications much work has been done on Herpetomonas, Crithidia, Leishmania, Trypanosoma, and other genera. By the application of the methods of Novy, the latter investigator and his students, and workers like Laveran, Franchini, Prowazek, Nöller, Wenyon, Kofoid, Fantham, Porter, McCulloch and others, paved the way for a scientific understanding of the true relationships of forms which ontogenetically and phylogenetically had seemed rather doubtfully interested in one another.

It therefore seemed to the writer that a restudy of Crawley's large species might contribute some facts not so easily obtained from smaller forms. *Trypanosoma americanum* is not only a very large species, but it is easily obtained and grown. It occurs around Washington, as Crawley found, in the blood of 74 per cent. of the cattle. The present writer's results on the incidence of infection in New Jersey are not comparable with the total percentage given by Crawley, for the reason that the former's work was begun after the first of October, 1921, and Crawley showed that: "By October the trypanosomes have become much less abundant in the blood, and an animal negative in that month might readily have been positive in July or August." The other purpose of the present investigation was to disclose, if possible, a few facts bearing on the parasitic or pathogenic possibilities of *T. americanum* and the manner of transmission from cow to cow.

During the months of October, November and December, cow blood was drawn under sterile conditions from the jugular vein at intervals. Following the cultural directions of Crawley, tubes were prepared containing 6 cc. of the defibrinated cow blood to be tested, above which was placed 6 cc. of sterile bouillon. The tubes were incubated at room temperature and the upper layer of erythrocytes examined for trypanosomes at the end of 3 or 4 days.

The accompanying table shows the number of cases that proved positive for trypanosomes.

Cultural Requirements.—As Crawley showed, the trypanosomes are so rare numerically in cattle that the preparation of cultures offers the only safe method for determining their presence. However, the present writer centrifuged 50 cc. of fresh defibrinated blood from cow 584. The blood from this cow had previously yielded cultures of trypanosomes. After centrifuging, the bottom layer was examined in a fresh state on several slides with a low power objective. After a long search one *T. americanum* was found. This specimen was very large and morphologically similar to the forms found in early cultures, 12 to 48 hours. The undulating membrane was short and ended anterior to the center of the body. No other specimens were found although others were doubtless present for the same blood from which the samples for centrifuging were removed yielded cultures later.

Table Showing Incidence of Infection in Cows and Yearling Heifers

Date	Cow	Results
1921		
Oct. 15	584	+ +
Oct. 15	600	— —
Oct. 15	601	+ +
Oct. 25	584	+ +
Oct. 25	600	— —
Oct. 25	601	+ +
Nov. 8	3750	— —
Nov. 8	843	+ +
Nov. 8	45	— —
Nov. 8	3877	— —
Nov. 8	859	— —
Nov. 8	3881A	+ +
Nov. 8	878	+ +
Nov. 8	8858	— —
1921		
Nov, 8	Calf 938	— —
Nov' 10	735	— —
Nov. 10	722	— —
Nov. 10	757	— —
Nov. 10	896	— —
Nov. 10	758	— —
Dec. 12	1096	— —
Dec. 12	1092	— —
Dec. 12	1085	— —
Dec. 12	1091	— —
Dec. 12	1075	— —
Dec. 12	1064	— —
Dec. 12	1063	— —
Dec. 12	1077	— —

Six cc. defibrinated blood were used for each culture. Two cultures were prepared from each sample of blood.

The cultural requirements and characters of *T. americanum* in liquid media were fully described by Crawley. The writer may add that he had just as good success with horse blood as when cow blood was used. However, after 5 or 6 days the trypanosomes begin to hemolyze the top layer of erythrocytes and it becomes difficult to see the colonies at the junction of the red cells and bouillon. This phenomenon does not appear to occur so rapidly with the cow blood cultures.* They remain clear for quite a long time and consequently one can see well enough to fish out individual colonies with a fine pipette.

The N. N. N. solid medium prepared either with cow or horse blood also proved highly satisfactory. As will be shown later, forms are obtained on this medium not found in liquid cultures. Moreover, the trypanosomes grow very rapidly on the N. N. N. medium during the first 4 or 5 days, and consequently it is a favorable source for the demonstration of dividing individuals. In applying it, however, care must be taken to keep the surface moist by adding sterile horse or cow serum every few days or by placing the petri dishes in a humid atmosphere. Colonies do not develop on the N. N. N. medium; the growth is diffuse. In order to obtain the best results all the cultures should be kept at room temperature; the higher incubator temperatures, 35 to 37 C. inhibit growth and produce degeneration and death in 3 to 5 days.

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* It is important to use fresh blood for culture media.

Cytology of the Stages Found in Cultures

In the study of this phase of the subject preparations were made from the various cultures at intervals. Some of these preparations were studied in the fresh condition or with intravital dyes. Others were fixed and stained. Crawley dried his films, staining them later with Wright's stain. The writer repeated this procedure, but abandoned it in favor of the "wet method," for the reason that much better and more beautiful preparations resulted. As a general routine, the films were drawn out very thinly and then immersed, while still wet, for 15 minutes in half and half absolute alcohol-ether mixture. They were then stained by Novy's modification of the Romanowsky method. This gave by far the best results, although certain cytologic structures could be better elucidated by using Schaudinn's fixative and Heidenhain's iron hematoxylin. Mallory's chloride of iron hematoxylin method was also used to advantage in a few cases.

The cultures are best studied 3 days after incubation at room temperature. At this time a sufficient number of trypanosomes have multiplied so that one has little difficulty in finding them. In 3 days the bodies of *T. americanum* averaged 17 to 18μ long. The free part of the flagellum was usually as long as the body or slightly longer. Often enormous forms were encountered. Some of these measured 25μ long or longer, exclusive of the flagellum.

In trypanosomes taken from liquid cow blood cultures 3 days old (Figs. 1, 2, 3, and 4), the body is long, more or less cylindrical and rather flexible. The flagellum on reaching the anterior part of the body becomes an undulating membrane which follows the length of the body laterally for about one-third or less of the distance. It then loses itself in the body of the trypanosome ending (Figs. 1 and 2) near a dark staining granule, the parabasal body. No blepharoplast or thickening of the end of the thread itself can be detected. The nucleus, when demonstrated, is located centrally (Fig. 1) or anterior to the central (Fig. 2). A karyosome is usually clearly differentiated. The end of the undulating membrane may be some distance from the nucleus (Fig. 1) or the two may lie closer together (Fig. 2). In many specimens large and deeply staining granules are distributed throughout the finely granulated cytoplasm. When these granules lie in close proximity to the end of the undulating membrane, it is difficult to determine which one constitutes the parabasal body. However, specimens such as the one represented in figure 2 are frequently found. In these cases only one large deeply staining body is found in the cytoplasm; it is in close proximity to the end of the thread and is consequently interpreted as the parabasal body.* Cases were found (Figs. 3 and 4) in which no nucleus was visible (unless one interprets the large deeply staining granules near the center of the body as such). These cases are difficult to interpret because they were present on slides containing other trypanosomes with well fixed and differentiated nuclei.

In specimens found in 8 day liquid cow blood cultures (Figs. 5 to 9), nearly all individuals have lost the undulating membrane. Many of the organisms have approached the typical herpetomonad type. Figure 5 represents such a case; no undulating membrane, rigid body with the nucleus in an extreme anterior position. Figures 6 and 7 represent bulbous forms with flagella; figures 8 and 9 more extreme types with that organ lacking. In figure 9 nuclear division seems to have occurred. (The writer doubts whether this is a futile attempt at endogenous budding.) Figure 10 represents one of several organisms found in an 8 day horse blood culture showing that some of the bulbous forms are still capable of reproduction.

Forms found in 14 day old cow blood cultures (Figs. 11 to 19) show bulbous types with flagella, and round or oblong ones without that organ and not simulating flagellates in the least. The discovery of such forms as those shown in figures 15 to 18 aroused the writer's suspicions that he may be confronted with the result of a kind of endogenous budding similar to that found by McCulloch (1919) in *Crithidia cmryophthalmi.* A diligent hunt revealed other stages such as that represented by figure 20 from 14 day old cow blood culture, and figure 21, found in a 20 day old cow blood culture. These stages are very rare and the writer has studied his cultures enough to feel sure that they do not develop, but abort. These structures seem to hint at either a phylogenetic recapitulation, or at certain latent developmental possibilities, and if any stages in the invertebrate host exist and were known a further expression of opinion might be possible.

In 20 day old cow blood cultures many degenerating forms appear. After 20 days the cultures gradually die out although a few forms may persist for a much longer time. Figures 22 to 23 represent two unusual

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[*] The nomenclature of Kofoid, Swezy and McCulloch is followed in describing the extranuclear organelles.

forms seen in a 20 day old cow blood culture. In figure 22 a short undulating membrane exists ending definitely at a well defined parabasal body which is here situated alongside of the nucleus. In figure 23 the nuclear system seems to have migrated posteriorly. On the N. N. N. medium with either cow or horse blood development is much more rapid and consequently degeneration occurs much sooner. The life of the trypanosomes on the solid medium is not nearly so long as is the case when it is grown in the blood bouillon mixture.

On 4 day old cultures grown on the N. N. N. plates such forms as those on Plate III appear. Figures 24 and 25 show types similar to those found in early liquid cultures with the exception that the organisms are smaller and the body more attenuated. Figures 26 and 27 represent respectively a large and a small herpetomonad type. Due to the rapid multiplication on the N. N. N. medium one readily obtains many division stages. Figure 28 shows nuclear and cell division, but the parabasal body is still intact. Figure 29 shows another form undergoing nuclear division. Figure 30 demonstrates nuclear, parabasal body, and cell division. Figure 31 represents another form of division superficially simulating constriction. There is no evidence for any other common method of division excepting longitudinal, therefore what happened here was partial longitudinal division accompanied by a movement of the two halves in opposite directions before final separation occurred. Figure 32 shows what appears like an abnormality, cell division without any apparent preceding nuclear division. Figure 33 represents a group of trypanosomes including actively growing and dividing forms, and senile and degenerating individuals. Figures 34 and 35 represent still other conditions encountered.

An examination of the N. N. N. plates in 7 days shows many curious shapes and forms that can only be interpreted as degeneration stages. In 9 days the degeneration stages are well accentuated and the protozoa have a tendency to become very large and amoeboid as shown in fixed and stained preparations. No amoeboid activity can, however, be determined in fresh films examined at room temperature or on the warm stage. Plate IV shows a series of degeneration forms. The appearance is strikingly like that of an amoeba. The cells are large with reticulated cytoplasm containing often many deeply staining granules. Figure 36 still possesses its flagellum, a telltale brand of the organism's flagellate origin. The nucleus has a well defined karyosome and chromatin granules within the clear nuclear substance. Figures 37 and 38 also show large nuclei filled with chromatic material, and figure 39 shows a karyosome as well. Figure 40 shows a long attenuated form. The cells although senile are still in some ways physiologically functional. The nuclei, as has been shown, are normal and the organisms are still at times capable of reproduction (Fig. 41). After about 12 to 14 days, however, the protoplasm of the organisms becomes coarsely granular, Brownian movement is seen within, and complete disintegration soon follows.

Specificity

Since *Trypanosoma americanum* occurs as a parasite in American cattle in which it produces no visible pathologic conditions, little hope was experienced that the inoculation of laboratory animals would throw any light on the nature of this parasitism. Nevertheless, two guinea pigs, one rabbit, six white and two wild mice were inoculated with young, 3 to 4 day old, cultures. Intraperitoneal inoculation was practiced on the guinea pigs, intravenous on the rabbit and subcutaneous, intraperitoneal and intravenous inoculation on the mice. Blood smears were made from time to time from all animals with negative results. Temperatures and weights demonstrated nothing.

One guinea pig was autopsied in 3 weeks. All the organs were carefully examined and cultures made with negative results. The mice, which were given from ¼ to 2 cc. of the cultures, dependent on the manner of inoculation, were all autopsied at intervals from 4 days to 2 months. Smears and cultures were made from the peripheral and heart blood, the spleen, liver, kidneys, and bone marrow, but no trypanosomes or stages of trypanosomes were found in smears or recovered in cultures. These results, taken in conjunction with the observations already published on cattle by Crawley, seem to indicate that *T. americanum* is specific to cattle.

It appeared to the writer that an invertebrate host, viz., some insect, might act as the transmitter of *T. americanum*. The insect fauna occurring around the cattle that harbored trypanosomes was carefully studied and only three forms appeared worthy of consideration, namely, *Stomoxys calcitrans, Haematobia serrata,* and *Musca domestica*. It may seem unnecessary to include the latter species, but it has been shown that the house fly will often "follow up" the bites of the true blood-sucking forms and under these conditions will engorge, so that consequently it may be a source of infection and transmission. Moreover, Darling (1912) showed that the house fly is capable of transmitting *T. hippicum* to healthy mules.

Collections of the three flies were made from the bodies and stables of animals known to harbor *T. americanum*. These flies were carefully dissected and the entire alimentary canal including the crop, salivary glands, and Malpighian tubes was examined. Ninety-two house flies, 147 stable flies and 85 horn flies were thus investigated, but no trypanosomes were found. *Herpetomonas muscae-domesticae* was frequently encountered in house flies, but no other protozoa were seen in the two blood suckers. These negative results should not discourage the point of view, still held, that one of these three flies will prove to be responsible for the transmission of *T. americanum*.

In order to determine whether *T. americanum* could survive in flies, 20 Stomoxys and 10 Haematobias were fed with a vigorous liquid culture of this flagellate. Two Stomoxys and two Haematobias were dissected immediately after engorgement and trypanosomes were found in abundance, showing that the flies actually ingested the organisms. Four Stomoxys and four Haematobias were dissected in 24 hours. The flagellates were found in the intestines of all, but had experienced a considerable modification. Many herpetomonad types were seen; rigid, attenuated with no undulating membrane. In 48 hours four more Stomoxys, and the remaining Haematobias were examined. In two cases what appeared like a few degenerating flagellates were seen. All of the remaining Stomoxys were dissected in 72 hours and nothing was found. The flies during this time were well cared for and supplied twice daily with defibrinated horse blood which they ate. This experiment seems to show that Stomoxys and Haematobia might act as the transmitters of *T. americanum,* but that the transfer from host to host must occur within 48 hours. The natural mode of transmission remains problematical, however, until *T. americanum* has actually been found in the intestine or some other organ of a fly caught wild.

Modifying Influence of the Environment, and Taxonomic Position

Recently Nöller (1920) has cast doubt on the validity of the genus Crithidia and places this genus in synonymy with Trypanosoma. This investigator worked with crithidia-like organisms morphologically very similar to *Trypanosoma americanum*. Nöller showed that trypanosomes could be changed into Crithidia and vice versa. He subjected the sheep trypanosome, growing on sheep blood agar, to different temperatures. At 30 C. the organism grew as a Crithidium with a short undulating membrane. At 37 C., in a few days, the body became snake-like, the rigid hind end became flexible and intermediate stages between Crithidia and true blood trypanosomes began to appear.

In the case of the trypanosomes of birds the same phenomenon occurred. At room temperature the organisms grew as Crithidia, but when subjected to 37 C. for 48 to 72 hours true trypanosomes appeared, although multiplication ceased at that temperature. If the plates were returned to room temperature rapid multiplication resumed in 12 to 48 hours, and the organisms changed again into Crithidia. Nöller also states that Trautmann succeeded with difficulty in converting the crithidial phases of *T. theileri* (an organism parasitic in European cattle) into trypanosomes by subjecting them to a temperature of 37 C. for from 5 to 8 days. From these experiments Nöller concludes that the genus Crithidia is a synonym for Trypanosoma.

Since *T. americanum* is morphologically really a Crithidia the writer repeated Nöller's experiments with this form. The experiments are given in detail below.

1. *Trypanosoma americanum* was grown on cow blood agar (2 plates) at room temperature 3 days. At this time an examination showed many active Crithidia with anterior nuclear complex and short undulating membranes. The plates were then placed in incubator at 37 C. and examined in 2 days. Multiplicative energy had ceased and among Crithidia many deformed individuals were found, such as bulbous and round forms. No trypanosomes were seen. In 4 days the organisms were found dead and disintegrating with the exception of a few greatly deformed individuals. This experiment was repeated at 35 C. with a similar result. No trypanosome types ensued.

2. *Trypanosoma americanum* was grown in liquid cow blood bouillon mixture (4 tubes) at room temperature for 3 days. At this time an examination showed active Crithidia with the anterior nuclear complex and short undulating membranes. The tubes were then placed in the incubator at 37 C. One day later the cultures were examined. Multiplication was inhibited. The organisms were crithidia-like with anterior nuclear system and short undulating membranes. The cultures were again examined in 2, 3, 4, and 5 days, but no conversion into true trypanosomes occurred. Deformed and degenerating individuals began to appear and in 6 to 7 days the cultures had "died out."

This experiment was repeated at 35 C. with a similar result. No trypanosome types ensued.

It might be well to state that all of these cultures were not only studied in the fresh state, but all results were checked with fixed stained preparations.

The above experiments prove that by the method employed the crithidia-like *T. americanum* was not converted into a true trypanosome.

The writer has never seen *T. americanum* approach the trypanosome type under any conditions. In cultures, as was shown above, herpetomonad types are common. These types were also observed, as was shown with the fly experiment, if the crithidial forms are introduced into the invertebrate intestine. Moreover, the examination of very early cultures made from cattle blood always revealed the crithidial type as both Crawley and the writer found. Lastly, the experiment with the freshly drawn and centrifuged blood mentioned in the beginning is also proof of the fact that the crithidia-like phases of *T. americanum* obtained in cultures resemble the forms as they occur within cattle.

It would seem then that the organism under discussion is a Crithidia and not a trypanosome. For morphological reasons this would seem to be the case. In the trypanosomes, the extranuclear organelles are at one time in the natural life cycle, or on some medium, or at some temperature according to Nöller, located posteriorly to the nucleus. Such forms have a well developed undulating membrane. These characters are common to trypanosomes found in the blood. *T. americanum* occurs naturally in the blood, yet the blood forms do not possess these characters nor can they be experimentally induced to assume them. In common with most Crithidia the nucleus is usually centrally located or anterior to the center. The extranuclear complex is located anterior to the nucleus and a short undulating membrane exists.

In spite of the crithidial nature of *T. americanum,* the writer feels it best not to include this flagellate in the genus Crithidia. The true Crithidia are forms inhabiting the alimentary tract of invertebrates, often in the intestines of plant-feeding insects. Such flagellates have never had occasion to come in contact with vertebrate blood and it would be impossible to predict what might happen to them should this occur. Since the organism under discussion resembles a Crithidium morphologically, but naturally lives in the blood of cattle, it seems best to regard it as an intermediate evolutionary stage between the true crithidians and true trypanosomes. For this reason, and on account of the fact that Crawley's organism has remained in the genus Trypanosoma so long, the writer prefers not to alter its taxonomic status.

In conclusion I wish to state that I am indebted to Dr. Ralph B. Little of this department for collecting the samples of blood.

SUMMARY

Trypanosoma americanum was successfully grown in horse blood medium and on the N. N. N. medium, as well as in cow blood medium. Development in the culture media was traced, and the cytological details of the various stages described.

T. americanum is specific to cattle. In freshly drawn blood and in very early cultures *T. americanum* resembles the majority of the forms found in 3 and 4 day old cultures.

Morphological and experimental data are presented to show that *T. americanum* is structurally a Crithidium. Prolonged culture and environmental alterations have a tendency to produce herpetomonad types but never trypanosome types.

Reasons are presented in support of the view that *T. americanum* is an intermediate evolutionary stage between true Crithidia and true trypanosomes. The name *Trypanosoma americanum* is retained.

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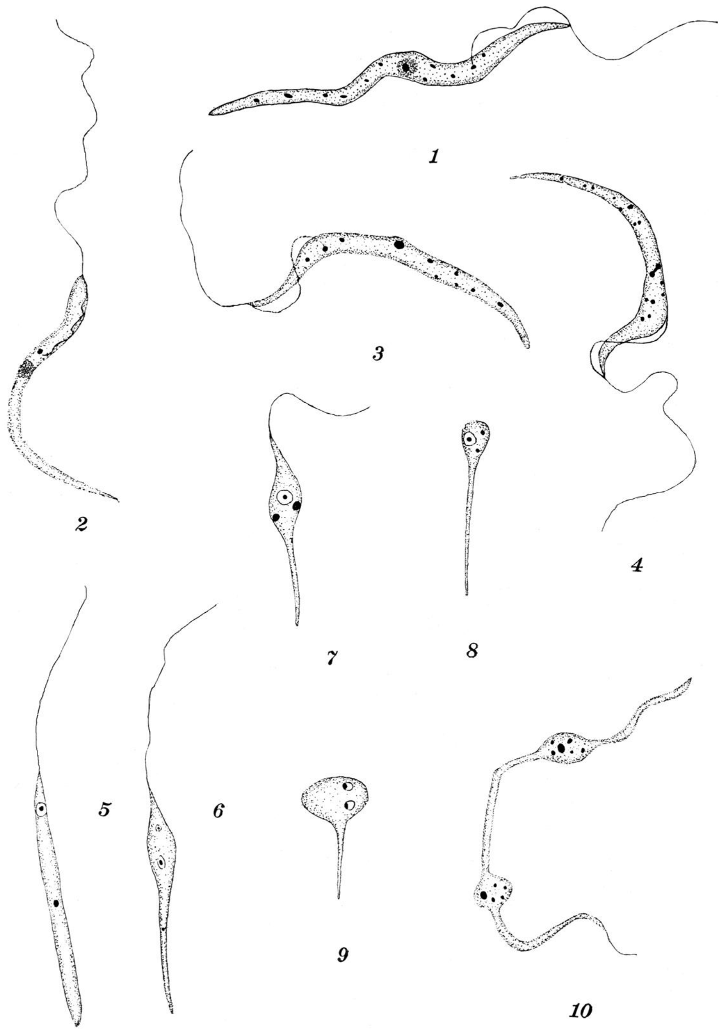


PLATE XIV

All figures represent *Trypanosoma americanum.* × 813.

Figs. 1-4.—Trypanosomes from 3 day liquid cow blood cultures. Figs. 5-9.—Specimens from 8 day liquid cow blood cultures. Fig. 10.—Specimen from 8 day liquid horse blood culture.

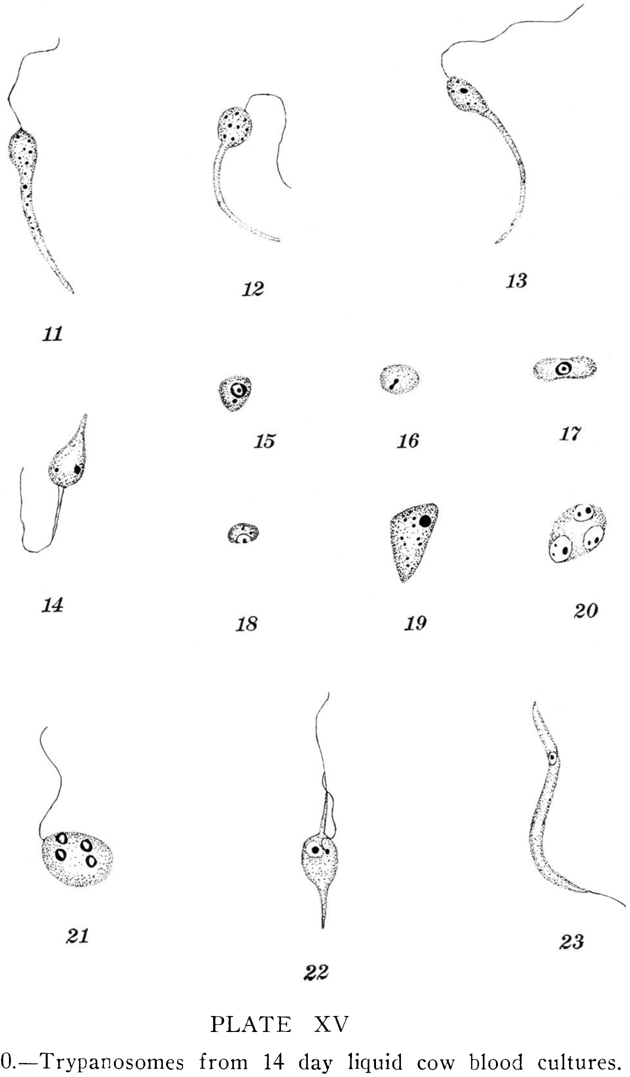


PLATE XV

Figs. 11-20.—Trypanosomes from 14 day liquid cow blood cultures. Figs. 21-23.—Specimens from 20 day liquid cow blood cultures.

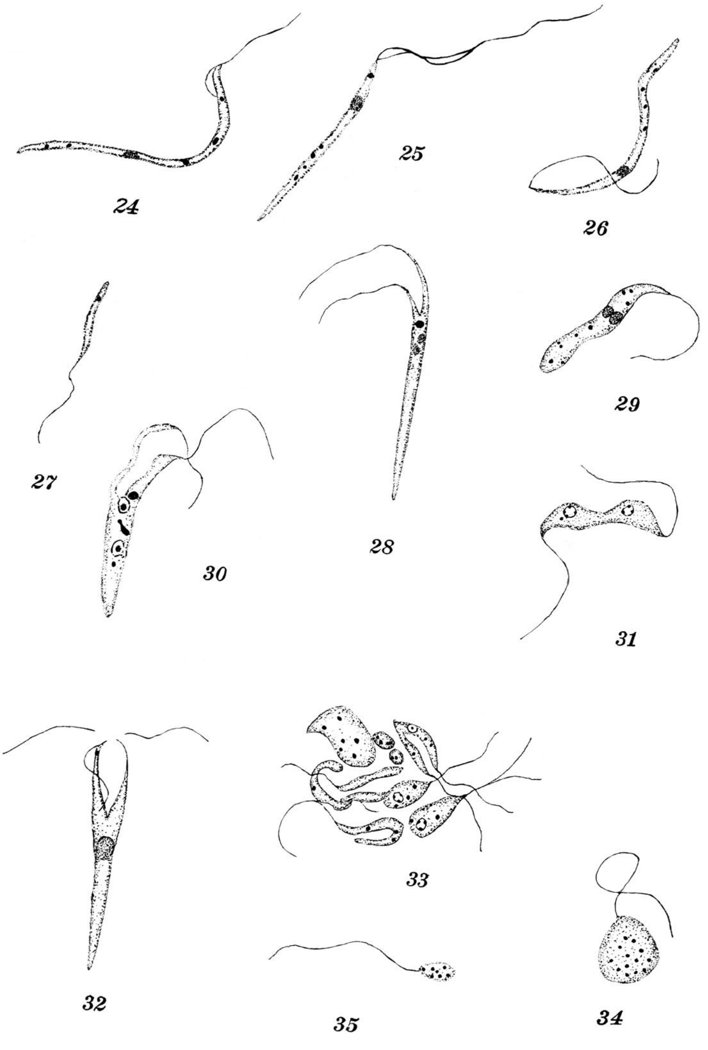
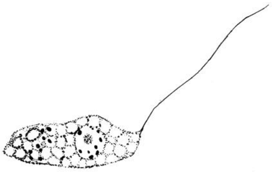


PLATE XVI

Figs. 24-35.—Trypanosomes from 4 day N. N. N. cultures.



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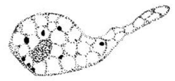

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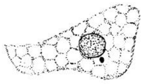

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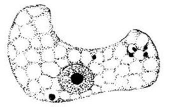

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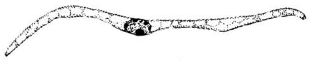

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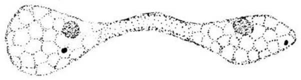

PLATE XVII

Figs. 36-41.—Trypanosomes from 9 day N. N. X. cultures.